

Exhibit A

APPLICATION OF THE PAPER DISC TECHNIQUE TO THE
COLLECTION OF WHOLE BLOOD AND SERUM SAMPLES
IN STUDIES ON EASTERN EQUINE
ENCEPHALOMYELITIS

LARS KARSTAD, J. SPALATIN* AND R. P. HANSON

From the Departments of Veterinary Science and Bacteriology, University
of Wisconsin, Madison, Wisconsin

The paper discs used in standardization procedures were (12.5 mm in diameter) of the type used in antibiotic sensitivity tests.† A disc 15 mm in diameter was used in some of the earlier work. In the laboratory saturation was accomplished by dropping serum from a pipette onto discs in petri plates. Saturated discs were exposed to the air at room temperature for several hours until dried, at which time the plates were covered and held without refrigeration until used.

The test procedure used was similar to that described in initial development of the technique.‡ Dried paper disc samples were placed in 0.6 ml volumes of sterile nutrient broth to allow the antibodies contained to elute. After a short

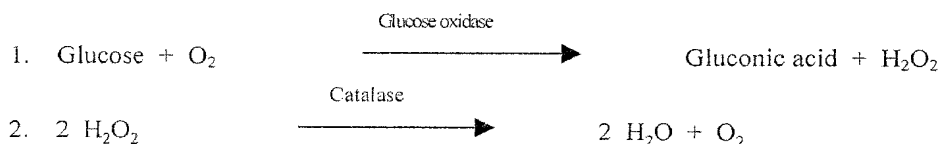
Exhibit B

Oxygen Transport Function

Dressings in accordance with the invention can act as efficient transporters of oxygen from the ambient atmosphere to a wound site. Oxygen from the ambient atmosphere is converted to hydrogen peroxide by the action of the oxidoreductase enzyme. Hydrogen peroxide is much more soluble in water than is molecular oxygen, so hydrogen peroxide transport through the dressing is generally much more efficient and rapid than that of molecular oxygen. When the hydrogen peroxide encounters catalase, which is naturally present in the wound, it decomposes to oxygen and water. In this way, oxygen is transported through the dressing in the form of hydrogen peroxide far more efficiently than transport of molecular oxygen.

An important feature of the dressing is that the dressing is of layered construction, including an upper layer (the first dressing component) (to be located remote from the skin in use) comprising oxidoreductase enzyme in dry condition, and a lower layer (the second dressing component) including a source of water and optionally a supply of glucose. The dressing in use functions to produce hydrogen peroxide in the first component or at the interface between the components, with the hydrogen peroxide diffusing through the second component and reacting to generate oxygen (in dissolved form) at the skin or wound surface, catalysed by catalase present at the skin surface and in wound fluid. This effect is explained on pages 16 and 17 of our specification. The effective transport of oxygen across the dressing in this way is very important and has beneficial effects for healing. This oxygen transport does not occur with the dressings disclosed in the prior art.

The generation of oxygen achieved with the dressings of the invention is based on two consecutive chemical reactions occurring in two stratified layers, as follows:

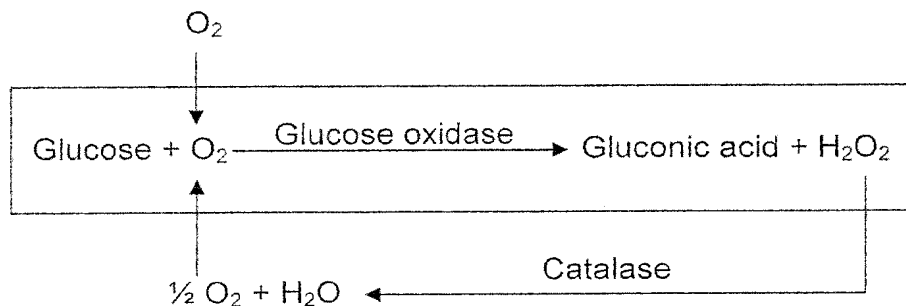


It is essential that the reactions are spatially separated in the system, with the first one in the first dressing component (away from the wound surface) and the second in the second dressing component or at the wound contact surface of the second dressing component.

In the first reaction, glucose from the wound site or the lower layer diffuses into the upper layer and reacts with oxygen from the surrounding atmosphere, catalysed by the enzyme glucose oxidase immobilized in the first dressing component, resulting in production of hydrogen peroxide. The hydrogen peroxide diffuses through the lower layer, and in the second reaction undergoes a reaction catalysed by catalase present at the skin surface and in wound fluid, resulting in production of oxygen. We refer to this effect as active oxygenation.

However, if glucose oxidase, glucose and catalase are mixed in a single system, e.g. in an aqueous solution or in a single hydrogel layer, no active oxygenation can occur. The

reason for this is that the oxygen generated in the second reaction above is converted back to hydrogen peroxide, catalysed by glucose oxidase. The reactions can be represented as follows:



The oxygen generated in the second reaction and also oxygen from the skin surface or wound bed thus reacts to form hydrogen peroxide, resulting in depletion of oxygen levels at the skin surface, which is disadvantageous for wound healing. Such single layer systems thus result in active deoxygenation of skin or wounds, in contrast to the active oxygenation achieved with the dressings of the invention. This effect arises because the glucose oxidase is adjacent to the skin, while in the dressings of the invention the glucose oxidase is kept remote from the skin by the intervening second dressing component.

WO 01/28600 (Green) discloses anti-infective wound dressings including dry glucose oxidase, dry lactoperoxidase and an iodide salt in a polymeric matrix. The dressing relies on use of water in body fluids for hydrating the enzyme (page 7, line 1). The dressings typically generate iodine, and are mainly of monolayer construction. All of the examples of WO 01/28600 concern single layer constructions. Figure 1, which is described on page 10, shows a mono-layer wound dressing comprising an acrylamide sheet with entrapped glucose oxidase, lactoperoxidase and iodide salt. Glucose diffuses into the layer from the wound site and oxygen diffuses in from the surrounding atmosphere, resulting in generation of iodine (see page 8, line 21). There is no reference to generation of oxygen at the wound site. Indeed, for the reasons explained above, active deoxygenation of the wound site would occur, with oxygen being drawn from the wound site to undergo glucose oxidase-catalysed reaction to form hydrogen peroxide.

WO 01/28600 does also disclose the possibility of multilayered dressings, with different layers having different compositions. See particularly page 6, lines 17-25 and Figure 2 which is described on pages 12-14. The Figure 2 embodiment includes an upper layer 21, remote from the wound in use, comprising iodide and an oxidant and a second lower layer 21 containing a proton source. The proton source is not specified. Possible proton sources include oxidoreductase (e.g via gluconic acid generated by glucose oxidase), a lipase, an esterase and weak acids (page 9, lines 5-6). The Figure 2 dressing is used with the upper, iodide-containing layer remote from the wound: see page 13, line 30 and page 18, line 15. The dressing is thus inverted as compared with the dressing of the invention and so would not have the active oxygenation function. If the prior art dressing included

an oxidoreductase enzyme in the lower layer, this would have the undesirable effect of withdrawing oxygen from the skin surface/wound site, as explained above.

The third paragraph on page 18 of WO 01/28600 discusses the possibility of a bilayer hydrogel dressing formed by compression of two hydrogel sponge layers: a top layer containing iodide and a lower layer containing oxidising agents. On wetting, in use, the hydrogel solvates, producing a gel solution (page 18, line 13) in which all of the ingredients will be more effectively mixed in a single layer. This will function as the monolayer embodiment discussed above, and so would not result in active oxygenation.

Exhibit C

Insense Technology Briefing. October 10th 2007

The Impact of peroxidase activity when included in the dual layer stratified enzyme system of Oxyzyme.

Background.

Insense wound healing technology uses a stratified dual layer wound (or skin) dressing, in which an oxidase enzyme is confined in a first pre-hydrated layer and the substrate is carried in a second pre-hydrated layer. Optionally, iodide ions can be included with the substrate in the second layer (Figure 1).

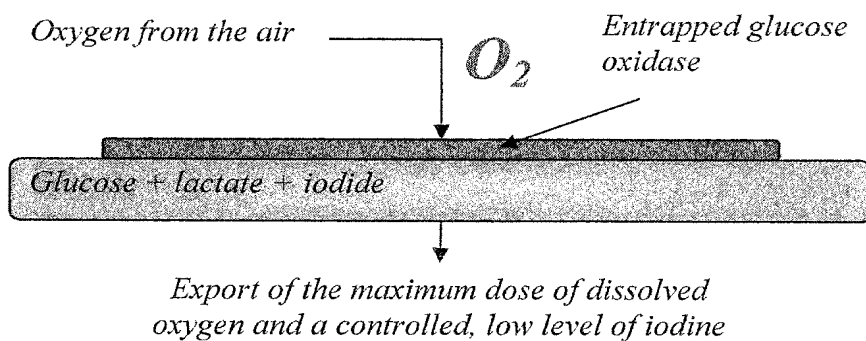


Figure 1: The basic components of the Insense oxygen transport/concentrator system

When the layers are brought together so that substrate can diffuse towards the enzyme in the first layer, an enzyme reaction occurs in which hydrogen peroxide is generated at the interface between the air and the first layer. Oxygen is, for all practical purposes, insoluble in the bulk of both hydrated layers, because of its extremely low solubility in water.

However, hydrogen peroxide is very soluble in the hydrated layers, and it diffuses away from the area of maximum concentration (the region in which it was generated), driven by the concentration gradient.

If the second dressing also contains iodide ions, then some of the hydrogen peroxide will react with iodide to form iodine. Also, depending on the relative concentrations of the reactants, some of the iodide behaves as a catalyst, causing the hydrogen peroxide to be broken down to oxygen and water, whereupon the iodide returns to its un-oxidised, original state (Figure 2).

Some of the hydrogen peroxide (the amount depending on the specific conditions prevailing in the second layer) will reach the lower face of the layer (the interface with the wound or skin). As it leaves the dressing (by diffusion), any hydrogen peroxide immediately encounters catalase or catalase-like enzyme activities, causing it to instantly break down to water and oxygen.

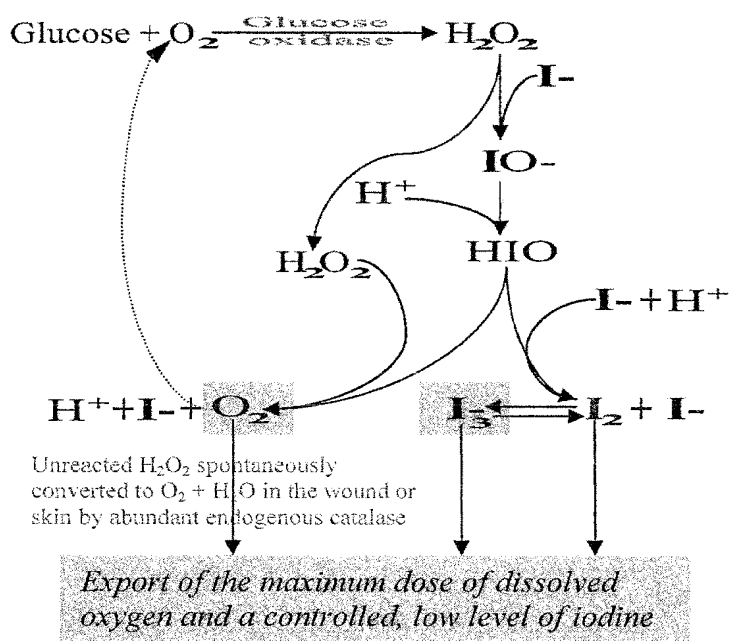


Figure 2: The chemical reaction scheme of the Insense oxygen transport/concentrator system

In the situation in which the second layer does not contain iodide ions, almost all of the hydrogen peroxide generated at the top interface (in contact with the air) will eventually reach the bottom interface (in contact with the wound or skin), but the quantity and rate of production is inherently controlled, such that the hydrogen peroxide-degrading capacity of the skin can continuously cause instant decomposition to oxygen and water.

These mechanisms are crucial to the Insense invention. The purpose is to transport and concentrate oxygen under the dressing, in the wound cavity or outer skin layers, optionally together with chosen levels of iodine. It has been found that unless the oxidase activity is confined to the top layer, no oxygen transport or concentration can take place and neither can iodine production occur. Apparently similar constructions make no provision for ensuring that the oxidase is confined to the top surface, in which case the transport/concentration mechanism would not work. In these other systems, however, oxygen transport/concentration was not an objective.

If the oxidase enzyme is applied in the opposite mode, wherein the enzyme layer is in contact with the wound or skin, the system will work in reverse, stripping oxygen out of the wound and delivering it into the air, leaving the wound or skin surface in a state of severe hypoxia.

It can also be appreciated that the inclusion, in either layer, of factors that interfere with these reactions will be detrimental to its function. For example, apparently similar formulations and devices described in other patents rely upon the inclusion of peroxidase activity to enable iodine production from hydrogen peroxide generated by glucose oxidase. In the Insense system, peroxidase activity in the second layer drives the production of iodine too strongly, by diverting the majority of the available hydrogen peroxide into the iodide oxidation route to generate iodine. In systems that are designed to contain peroxidase,

iodine production is the sole objective - oxygen transport/concentration is not. Oxygen transport in this way is unique to the Insense invention.

Thus, previously described systems, in which maximal iodine production is always the primary objective, clearly teach that peroxidase is a necessary ingredient. Without peroxidase, the system is inadequate for its purpose.

For the Insense invention, in contrast, it follows that inclusion of peroxidase in any part of the system must be avoided, if oxygen transport/concentration is to be sufficient for its purpose. Peroxidase in this situation would compete strongly for the available hydrogen peroxide and would, therefore, seriously compromise the oxygen transport.

Experimental strategy

The damaging effect of peroxidase on the Insense oxygen transport/concentration system is clearly shown in the following experiments, in which peroxidase was introduced in varying quantities to different parts of a stratified dual layer system, constructed from aqueous poly-AMPS hydrogel and based on glucose oxidase and glucose. The oxygen transport/concentration efficiency was determined by continuous measurement of the oxygen detectable at the lower surface of the dressing, by means of an oxygen electrode covered with a Teflon film dosed with a thin layer of catalase solution. This catalase dose would normally be supplied by the skin or wound, when in use. The test dressing was placed on the flat surface of the oxygen electrode in such a way that the electrode membrane interfaced with the dressing as would a wound or skin (Figure 3).

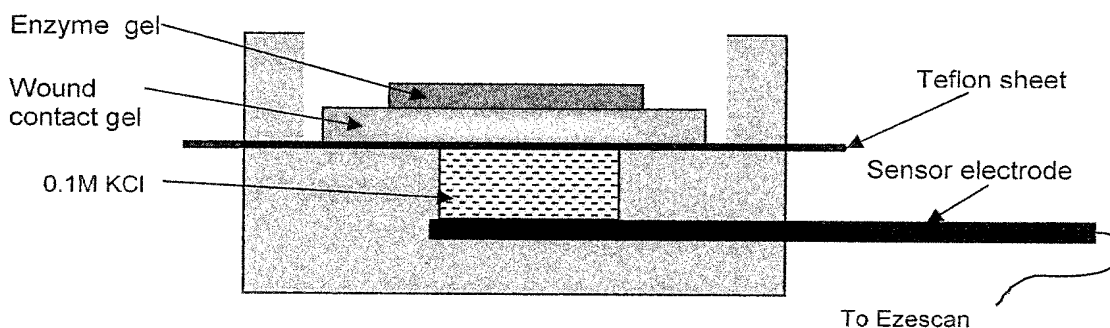


Figure 3: Diagrammatic representation of the electrochemical test rig used to continuously monitor oxygen or iodine export from Insense oxygen transport/concentration gel systems.

In one type of experiment, an aqueous solution of lactoperoxidase was applied between the enzyme gel (the first gel) and the substrate containing gel. In this way, the peroxidase was able to compete at a restricted site (the interface between the two gels) for the hydrogen peroxide as it diffused away from the point at which it was synthesised, toward the wound or skin interface (see Figure 4).

Location of instilled peroxidase solution



Figure 4: the location of the peroxidase solution

In a second type of experiment, an aqueous solution of lactoperoxidase was placed below the second layer (i.e. the layer containing the substrate for the oxidase), where the dressing would normally interface with the wound or skin (see Figure 5).

Location of instilled peroxidase solution



Figure 5: the location of the peroxidase solution in the second experiment

In a third type of experiment, various levels of lactoperoxidase were added to the formulation of the second layer, together with the substrate and iodide, so that the peroxidase was uniformly distributed throughout the layer (see fig 6).

Location of co-formulated peroxidase



Figure 6: the location of the peroxidase solution when it was co-formulated in the substrate layer

A fourth experiment was conducted in a way similar to that of the third approach, but with continuous sensing of iodine instead of oxygen, to show that the disruptive effect of the peroxidase was associated with a diversion of the reaction into the non-reversible production of iodine.

Methods

For the first two experiment sets, standard Oxyzyme gels were utilised, with lactoperoxidase solution added at the points shown in the diagrams (either the interface between the gels or

with the catalase solution below the activated gels). In order to mimic the wound or skin surface, 30ul of 0.1mg/ml catalase solution (=7 units) was placed onto the Teflon membrane above the electrode. In each experiment, a 2.8cm² piece of substrate gel was placed onto the catalase-dosed Teflon membrane and then a 2cm² section of standard Oxyzyme glucose oxidase gel was placed on top of the glucose gel to activate the system (figure 3).

For the experiment with gels containing uniformly distributed Oxyzyme, new, purpose-made gels were constructed with the following formulations:

Formulation of Hydrogels:

	(i)	(ii)
Na AMPS:	15%	15%
Amm. AMPS	15%	15%
Glucose	5%	5%
KI	0.05%	0.05%
LPO	0.5mg/ml	0.01mg/ml
Cross linker	0.19%	0.19%
Photo initiator	0.01%	0.01%

In each experiment electrochemical testing was continued for at least 1200 minutes.

Finally, electrochemical analysis for iodine production rates was conducted on the gels with lactoperoxidase uniformly incorporated in the substrate layer, in order to confirm that the peroxidase activity caused a disruptive bias away from the required oxygen transport and into iodine production.

Results

As shown in figure 7, the instillation of peroxidase solution into the Insense oxygen transport/concentration system caused a major decrease in efficiency. There was a substantial diversion of the chemical reaction (shown in figure 2) away from the oxygen transport route and into the iodine generation route. The position of the instilled lactoperoxidase determined the dynamics of the interference.

When the peroxidase was positioned between the gels (figure 4), it intercepted the hydrogen peroxide before it could disperse fully into the lower substrate gel. In this situation, the peroxidase-mediated suppression resulted in a long delay in oxygen export from the lower gel, allowing a prolonged period of relative hypoxia (about 150 minutes). Moreover, the total oxygen export was substantially lower than the amount exported from the preferred formulation.

When placed at the lower surface (figure 5), the hydrogen peroxide encountered the peroxidase after it had traversed the lower substrate gel. The peroxide flux had, therefore, been in prolonged contact with iodide ions. Even so, the oxygen export rate and magnitude was heavily suppressed. Incorporation of peroxidase at the low level of just 100µg within the whole of the substrate gel also caused heavy suppression of the oxygen transport mechanism. The extent of suppression was of a similar magnitude to that resulting from instillation below the dressing, *pro rata* to the peroxidase dose (100µg vs 300µg).

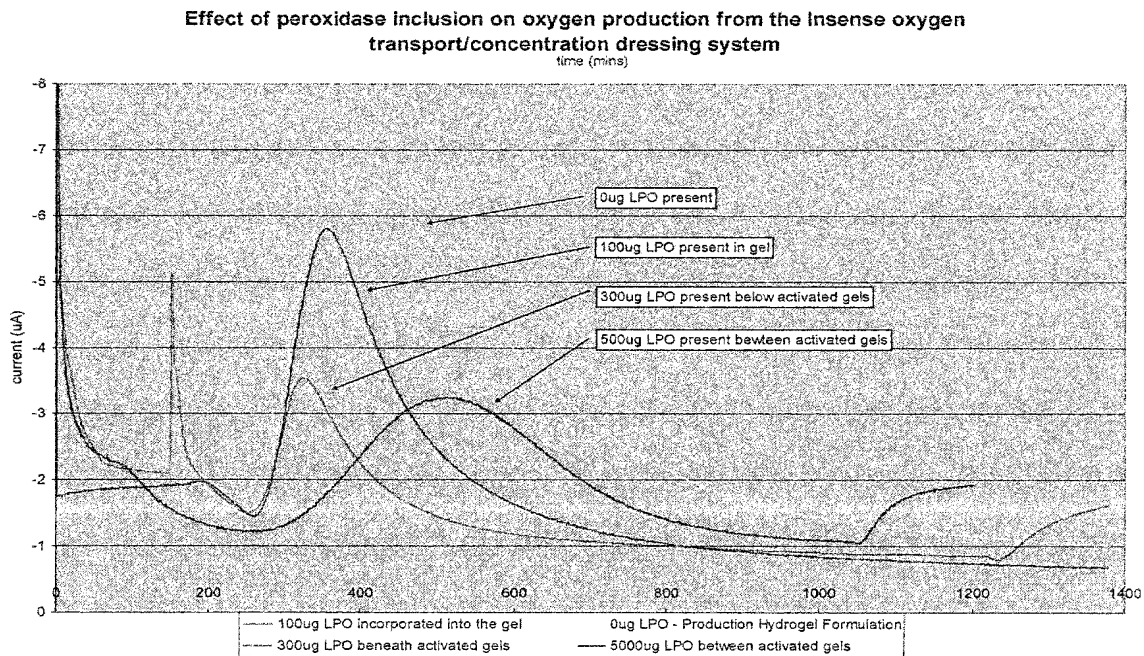


Figure 7: The impact of peroxidase inclusion on the oxygen transport through the Insense stratified oxygen transport/concentration system.

The iodine production curves (Figure 8) confirm that incorporation of peroxidase into the formulation diverts the reaction away from its intended route, by increasing the early production of a major iodine surge. These distorted iodine profiles show that peroxidase is an unacceptable ingredient, as it interferes with the reactions that are essential for the proper functioning of the invention and disrupts the required sustained, slow build-up iodine release.

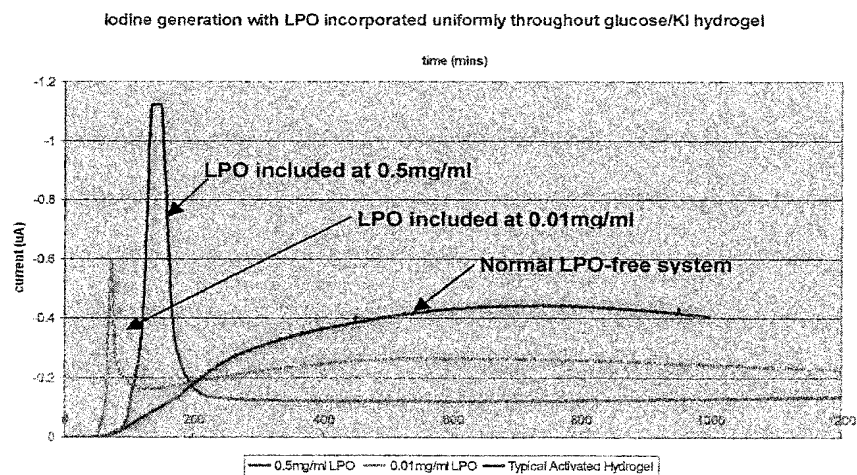


Figure 8: The impact of peroxidase inclusion on the generation of iodine within the Insense stratified oxygen transport/concentration system.

Conclusions

The mechanism of the present invention (stratified pre-hydrated layers with oxidase confined to the top layer), based on an enzyme-driven oxygen transport/concentrator system, requires that peroxidase is excluded. Peroxidase can not be included at any location within the stratified assembly, as it inevitably suppresses the oxygen transport mechanism. It also interferes with the slow, sustained-release iodine profile.

There is no benefit to be derived from inclusion of peroxidase, as the novel Insense configuration, surprisingly, allows the programmed, sustained export of iodine, without the use of such enzymes. In this unique situation, it is (also surprisingly) possible to control the dose-profile and magnitude of iodine export by means of other factors, such as depth of gel, water levels and extent of cross-linking.

Andrew Austin & Paul Davis
Insense Ltd